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The stabilization of β-catenin leads to impaired primordial germ cell development via aberrant cell cycle progression

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Abstract

Primordial germ cells (PGCs) are germ cell precursors that are committed to sperm or oocytes. Dramatic proliferation during PGC development determines the number of founder spermatogonia and oocytes. Although specified to a germ lineage, PGCs produce pluripotent embryonic germ (EG) cells in vitro and testicular teratomas in vivo. Wnt/β-catenin signaling regulates pluripotency and differentiation in various stem cell systems, and dysregulation of this signaling causes various human cancers. Here, we examined the role of Wnt/β-catenin signaling in PGC development. In normal PGC development, Wnt/β-catenin signaling is suppressed by the GSK3β-mediated active degradation of β-catenin and the low expression of canonical Wnt molecules. The effects of aberrant activation of Wnt/β-catenin signaling in PGCs were analyzed using mice carrying a deletion of the exon that encodes the GSK3β phosphorylation sites in the *β-catenin* locus. Despite the potential activity of Wnt/β-catenin signaling in stem cell maintenance and carcinogenesis in various cell lineages, teratomas were not induced in the mice expressing the nuclear-localized β-catenin in PGCs. Instead, the mutant mice showed germ cell deficiency caused by the delayed cell cycle progression of the proliferative phase PGCs. Our results show that the suppression of Wnt/β-catenin signaling is a prerequisite for the normal development of PGCs.

Keywords: Primordial germ cell; β-catenin; Wnt; Proliferation

Introduction

Wnt is a family of secreted proteins that regulate several physiological and pathological processes in the development and maintenance of various tissues (Logan and Nusse, 2004). Wnt/β-catenin signaling, which is activated by canonical Wnt molecules, controls cell proliferation and apoptosis. Aberrant activation of Wnt/β-catenin signaling leads to tumorigenesis in the intestine, skin, and mammary gland (Moon et al., 2004). In addition, Wnt/β-catenin signaling regulates the maintenance of various stem cell systems, including intestinal epithelial, follicular, hematopoietic, and embryonic (ES) stem cells (Huelsken et al., 2001; Reya et al., 2003; Sato et al., 2004; van de Wetering et al., 2002; Zechner et al., 2003). In contrast, non-canonical Wnt signaling participates in cell movements, such as convergent extension and planar cell polarity (Veeman et al., 2003).

Wnt signaling can be classified into two categories: the canonical Wnt/β-catenin and non-canonical pathways. β-catenin is a central signal transducer of Wnt/β-catenin signaling, whereas distinct intracellular signaling pathways are activated downstream from non-canonical Wnts (Logan and Nusse, 2004; Veeman et al., 2003). The stability of β-catenin is regulated by the so-called β-catenin destruction complex, which consists of glycogen synthase kinase-3β (GSK3β), adenomatous polyposis coli protein (APC), Axin, and casein kinase I. In the absence of canonical Wnt stimulation, β-catenin is phosphorylated by GSK3β and subsequently degraded via the

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ubiquitin–proteasome pathway. However, when canonical Wnts bind to frizzled (Fzd)-lipoprotein related protein (Lrp) 5/6 receptor complexes and activate Dishevelled (Dvl) proteins, GSK3β activity is inhibited, which leads to the accumulation of β-catenin in the cytoplasm and nucleus. The stabilized β-catenin forms heterodimers with T cell factor (TCF) transcription factors and regulates a variety of target genes.

Cell cycle status is strictly regulated in embryonic gametogenesis (McLaren, 2003; Wylie, 2000). In mice, the first germ lineage cells, which are called primordial germ cells (PGCs), differentiate from a population of epiblasts at embryonic day 7.25 (E7.25). PGCs migrate through the hindgut and dorsal mesentery and eventually populate the genital ridges until E11.5. Since PGCs proliferate actively during the migratory phase, the number of PGCs increases drastically from ~45 at E7.25 to ~25,000 at E13.0. In males, PGCs enter mitotic arrest in the G1/G0 phase at E13.5 and spermatogonia resume proliferation and undergo spermatogenesis. In females, PGCs enter meiosis at E13.5, and oocytes reach the quiescent stage at prophase of the first meiosis after birth. The defective proliferation during PGC development results in germ cell deficiency in some mutant mice (Atchison et al., 2003; Chabot et al., 1988; Geissler et al., 1988). Therefore, the proliferation of PGCs by E13.5 principally determines the numbers of founder spermatogonia in males and oocytes in adult females. However, the mechanisms that regulate PGC proliferation largely remain elusive.

PGCs possess the potential to regain pluripotency, although their developmental potential is restricted to germ cells in normal development. First, PGCs are the originators of testicular teratomas, tumors that contain collections of various differentiated and undifferentiated cells (Stevens, 1967). Second, pluripotent stem cell lines, embryonic germ (EG) cells, can be established from PGCs when PGCs are cultured in the presence of stem cell factor (SCF), leukemia inhibitory factor (LIF), and basic fibroblast growth factor (bFGF) (Matsui et al., 1992; Resnick et al., 1992). Previously, we demonstrated that phosphoinositide-3 kinase (PI3K) signaling is important for this process, using mice deficient in the tumor suppressor Pten, a lipid phosphatase that antagonizes PI3K (Kimura et al., 2005). Specifically, the enhanced activation of PI3K signaling by the absence of *Pten* increased the generation of EG cells and brought about testicular teratomas (Kimura et al., 2003b; Moe-Behrens et al., 2003). PI3K signaling exerts its biological effects through the activation of various downstream molecules (Brazil et al., 2004). Since PI3K signaling also induces the nuclear localization of β-catenin (He et al., 2004; Persad et al., 2001; Tian et al., 2004), β-catenin signaling is one of the candidates for regulating tumorigenesis and the stem cell properties of PGCs.

In this study, we examined the effect of aberrant activation of Wnt/β-catenin signaling in PGC development by analyzing mice that express the nuclear-localized mutant form of β-catenin in PGCs. Unlike *Pten*-deficient mice, the mice expressing nuclear-localized β-catenin did not show early teratoma onset, but exhibited abnormal cell cycle progression, which leads to germ cell deficiency.

Materials and methods

Mice

Male *TNAP-Cre* mice were mated with female heterozygous and homozygous *β-catenin$^{floxed\ exon3}$* mice to produce offspring expressing nuclear-localized β-catenin in PGCs (Harada et al., 1999) because Cre-mediated recombination in oocytes can be avoided when the *TNAP-Cre* locus is transmitted from the male (Lomeli et al., 2000). The *Oct-4-EGFP* mice are described (Yoshimizu et al., 1999). PGCs were purified from E13.5 gonads of the *Oct-3/4-EGFP* mice using FACS-Vantage (Becton Dickinson, Franklin Lakes, NJ, USA). The purity of the sorted PGCs, which was analyzed using an Alkaline Phosphatase Staining Kit (Sigma-Aldrich, Inc., St. Louis, MO, USA), exceeded 99%. The morning on which a copulation plug was found was defined as E0.5. The sex of the E11.5 embryos was determined using sex-chromosome-specific PCR (Chuma and Nakatsuji, 2001). Animal care was in accordance with the guidelines of Osaka University.

RT-PCR

Total RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using the ThermoScript RT-PCR system (Gibco BRL, Rockville, MD, USA) as described (Kimura et al., 2003a). The PCR products were analyzed using 2.0% agarose gel electrophoresis and visualized with ethidium bromide staining. The degenerate primer pair for amplifying canonical and non-canonical Wnt is described (Van Den Berg et al., 1998). The following gene-specific primer pairs were used for PCR amplification: *Wnt-1*, 5′-GCAAGGCCAGGCAGGCCATG-3′, 5′-CACTCACGCTGTGCAGGATC-3′; *Wnt-3a*, 5′-CGATGGCTCCTCTCGGATAC-3′, 5′-TGCTGACGGTGGTGCAGTTC-3′; *Wnt-8a*, 5′-GCAGGACCATGGGACACTTG-3′, 5′-GAAGGATGTCTCTCTCGTGG-3′; *Wnt-5a*, 5′-GGAAGGTGGGCGATGCCCTC-3′, 5′-TGCAATGACAGCGTTCGGTC-3′;*Wnt-5b*, 5′-GCAAGGTGGGGGACCGTTTG-3′, 5′-CACCTGAACGCTCTTGAAGC-3′; *Wnt-6*, 5′-ACGGCTGCTGGAGCGCTTCC-3′, 5′-TCTCCTCGAGCTGTACGCTC-3′; *Wnt-11*, 5′-CTGACCTCAAGACCCGCTAC-3′, 5′-CCACCACTCTGTCCGTGTAG-3′; *Lrp5*, 5′-AGCAAGTCCGTGATGAGCTC-3′, 5′-CCATACCTCGAATGACGTAG-3′; *Lrp6*, 5′-CGAGGCAAATCAATGATCAG-3′, 5′-GGTAGCTGTACGGCCTATAG-3′; *Fzd3*, 5′-GACAACATGGATCCAGGAAC-3′, 5′-CCCTTGAGTGGAAGTTCCTC-3′; *Fzd4*, 5′-CAGCCAGCTGCAGTTCTTCC-3′, 5′-GGTCCTTCCATGCACATGTG-3′; *Dvl1*, 5′-CCACCTGCTTACCAGGACCC-3′, 5′-AGAACTCACAGGGGTTTCCC-3′; *Dvl2*, 5′-CCACCCTACCACGAGCTTTC-3′, 5′-CTGCACTGCTGTGGAGACAG-3′; *Dvl3*, 5′-ACACCCAGGCTTCCCAGAAC-3′, 5′-AGCATCGGGGGACCATAGAG-3′; *GSK3β*, 5′-AGGCACATCCTTGGACAAAG-3′, 5′-TCAGGTGGAGTTGGAAGCTG-3′; *Axin1*, 5′-GTATGTGCAGGCAGTCATGC-3′, 5′-TCAGTCCACCTTTTCCACCT-3′; *APC*, 5′-GAAGAATAGCCAGGATCCAG-3′, 5′-TTAAGGAGAGCAGAACTGTG-3′; *TCF-1*, 5′-CAGGTGGCATGCACTATCTC-3′, 5′-AGCCTAGAGCACTGTCATCG-3′; *TCF-4*, 5′-CTTCTGGTAGCTCTGAGATC-3′, 5′-TTTCACATCTGTCCCATGTG-3′; *PGC7/Stella*, 5′-CAATCTTGTTCCGAGCT-3′, 5′-GCTTTCACATCTGCTGTGCG-3′; *β-actin*, 5′-GTGACGAGGCCCAGAGCAAGAG-3′, 5′-AGGGGCCGGACTCATCGTACTC-3′.

Immunohistochemistry

The testes and ovaries were fixed with 4% PFA and embedded in OCT compound (Sakura, Torrance, CA, USA). Frozen sections were cut at 7-μm thickness and stained as described previously (Kimura et al., 2003b). The primary antibodies used were antibodies against β-catenin (1:200; BD Transduction Laboratory, San Diego, CA, USA; 1:1000; Sigma), Ki67 (1:50; BD PharMingen, San Diego, CA, USA), BrdU (1:50; Roche, Mannheim, Germany), phospho-histone H3 (1:200; Upstate Biotech., Lake Placid, NY, USA) (Hendzel et al., 1997), PGC7/Stella (1:1000) (Sato et al., 2002), mvh (1:1000) (Toyooka et al., 2000), SSEA-1 (1:200; Kyowa Hakko, Tokyo, Japan), and TRA98 (1:1000) (Tanaka et al., 1997). Signals for these primary antibodies were visualized using the appropriate Alexa-Fluor-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA).

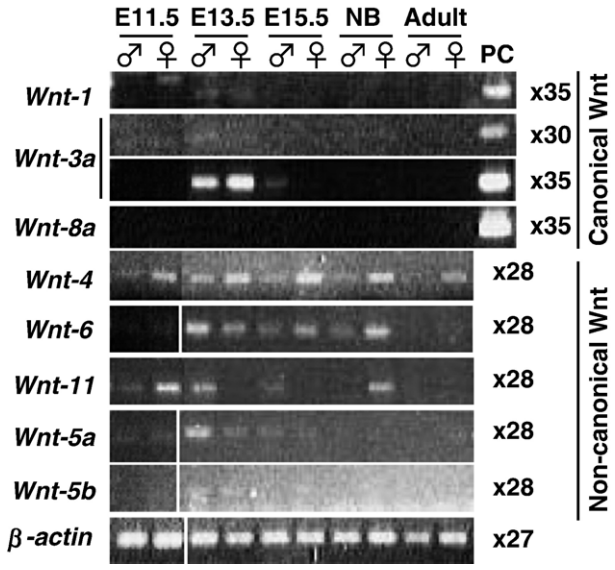

Fig. 1. Expression of canonical and non-canonical Wnt in testes and ovaries. The expression of Wnts mRNA in testes and ovaries at various developmental stages (E11.5, E13.5, E15.5, newborn (NB), and adult) as determined by RT-PCR with gene-specific primer pairs. Amplification cycles are indicated to the right of the panels. The sex of E11.5 embryos was determined using sex-chromosome-specific PCR.

Quantification of PGCs

Serial sections encompassing entire gonads were stained with the following antibodies to visualize PGCs: anti-PGC7/Stella antibody and SSEA-1 for PGCs at E10.5–12.0 and anti-mvh antibody for PGCs after E12.5. The sections were also stained with anti-β-catenin antibodies to determine the PGCs expressing the nuclear-localized mutant β-catenin. First, the section with the maximum number of PGCs was selected and then the adjacent four sections were collected every three sections to avoid counting the same cells. The number of PGCs in the five sections was counted.

Apoptosis and BrdU incorporation assays

PGCs were stained with LysoTracker-Red (Molecular Probes) as described (Atchison et al., 2003; Yao et al., 2002). Briefly, E12.0 gonads were dissected and cultured in 500 μl DMEM medium with 2 μl/ml LysoTracker-Red for 30 min in a 5% CO_2 incubator at 37°C. The gonads were then fixed with 4% PFA, and the frozen sections were stained with anti-PGC7 and anti-β-catenin antibodies. The TUNEL assay was performed using an In Situ Cell Death Detection Kit (Roche), according to the manufacturer's instructions. To label the cells at S phase, BrdU (50 μg/kg body weight) was injected intraperitoneally into pregnant mice. After labeling for 6 h, the gonads were collected and fixed with 4% PFA. Frozen sections treated with microwaves were stained with anti-BrdU antibody.

Results

Wnt/β-catenin signaling in germ cell development

First, we examined which Wnt family molecules were expressed in the gonads at E13.5. Wnt cDNAs were amplified from gonad RNA by RT-PCR using a degenerate primer set that is reported to amplify a variety of canonical and non-canonical Wnts mRNAs (Van Den Berg et al., 1998). Sequencing of the amplified cDNA clones revealed that non-canonical Wnts were expressed predominantly in both male and female gonads. In testes, 47 and 32% of the clones corresponded to *Wnt-6* and *Wnt-4*, respectively, while in ovaries, 67 and 17% were *Wnt-4* and *Wnt-11*, respectively. Canonical Wnt cDNA was not obtained in this analysis.

Next, the expression of canonical and non-canonical Wnts in testes and ovaries was analyzed at various developmental stages using the gene-specific primer sets. As shown in Fig. 1, *Wnt-4* was highly expressed in ovaries from E11.5 to adult and in the testes of embryos. The expression of the other non-canonical Wnt mRNAs, including *Wnt-6*, *Wnt-11*, *Wnt-5a*, and *Wnt-5b*, was also detected in the testes and ovaries of embryos and newborn mice. In contrast, the expression levels of canonical Wnts, such as *Wnt-1* and *Wnt-3*, were low and *Wnt-8a* was not detected at any developmental stage. Therefore, several non-canonical Wnts were strongly expressed, while canonical Wnts were hardly detected in the gonads throughout life.

The expression of intracellular Wnt/β-catenin signaling components in PGCs was examined using RT-PCR analysis. For this purpose, E13.5 PGCs were purified from transgenic

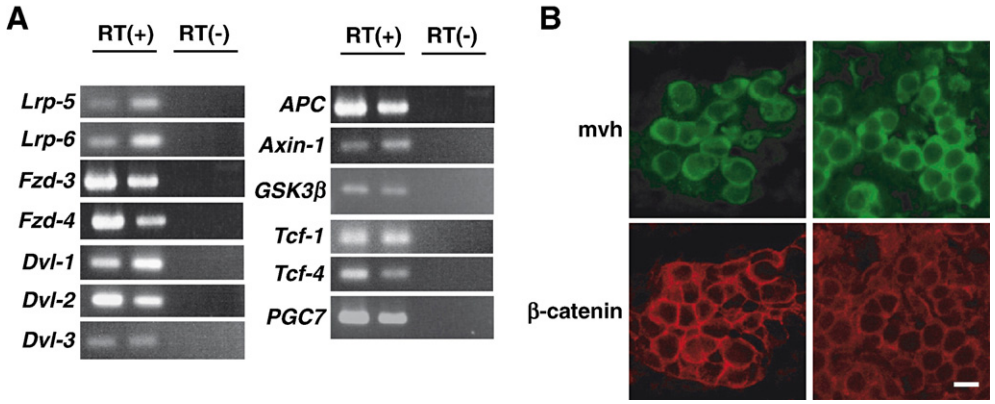

Fig. 2. Canonical Wnt signaling components in PGCs. (A) Expression of the mRNAs for the canonical Wnt signaling components in PGCs. PGCs were purified from E13.5 Oct-4-EGFP transgenic embryos at a purity exceeding 99%. Expression was analyzed using RT-PCR. (B) Subcellular localization of β-catenin in PGCs. The E13.5 gonads were stained with antibodies against the germ-cell-specific marker mvh (mouse vasa homolog: green) and β-catenin (red) and analyzed under confocal microscopy at 1-μm thickness. The β-catenin signal was predominant in the plasma membrane and cytoplasm, but not in the nucleus of male and female PGCs. Note that mvh is a cytoplasmic protein. Scale bar, 10 μm.

mice expressing EGFP under the control of the germ-cell-specific *Oct-4* promoter. Both male and female PGCs expressed mRNAs for Wnt receptors (Fzd-3, 4, Lrp5/6), signal transducer Dishevelled (Dvl-1, 2, 3), β-catenin destruction complex (APC, Axin-1, and GSK3β), and downstream transcription factors (Tcf-1, 4) (Fig. 2A). To examine subcellular localization of β-catenin in PGCs, we stained the gonad sections with antibodies against β-catenin and mouse vasa homologue (mvh), which is a germ-cell-specific and cytoplasm-localized protein. Immunohistochemistry of the E13.5 gonads showed that β-catenin was predominantly localized in the plasma membrane and cytoplasm, but not in the nucleus (Fig. 2B). Thus, the Wnt/β-catenin signaling pathway was not activated in PGCs because the expression of canonical Wnts was low in the gonads.

Germ cell deficiency in the stabilized β-catenin mutant mice after birth

To determine the effect of aberrant activation of Wnt/β-catenin signaling in PGC development, we generated mice that express the stabilized mutant β-catenin protein in PGCs. To do this end, we used *β-catenin*$^{floxed\ exon3}$ mice in which exon 3 of *β-catenin* containing the GSK3β phosphorylation sites is flanked with *loxP* sequences (Harada et al., 1999). The *β-catenin*$^{floxed\ exon3}$ mice were crossed with *TNAP-Cre* mice that express Cre recombinase in PGCs from the *TNAP* (*tissue nonspecific alkaline phosphatase*) locus (Lomeli et al., 2000). In the PGCs of stabilized β-catenin mutant (*β-catenin*$^{floxed\ exon3/+}$; *TNAP-Cre*) mice, exon 3 is deleted in one of the *β-catenin* loci and the GSK3β-resistant β-catenin protein is synthesized from

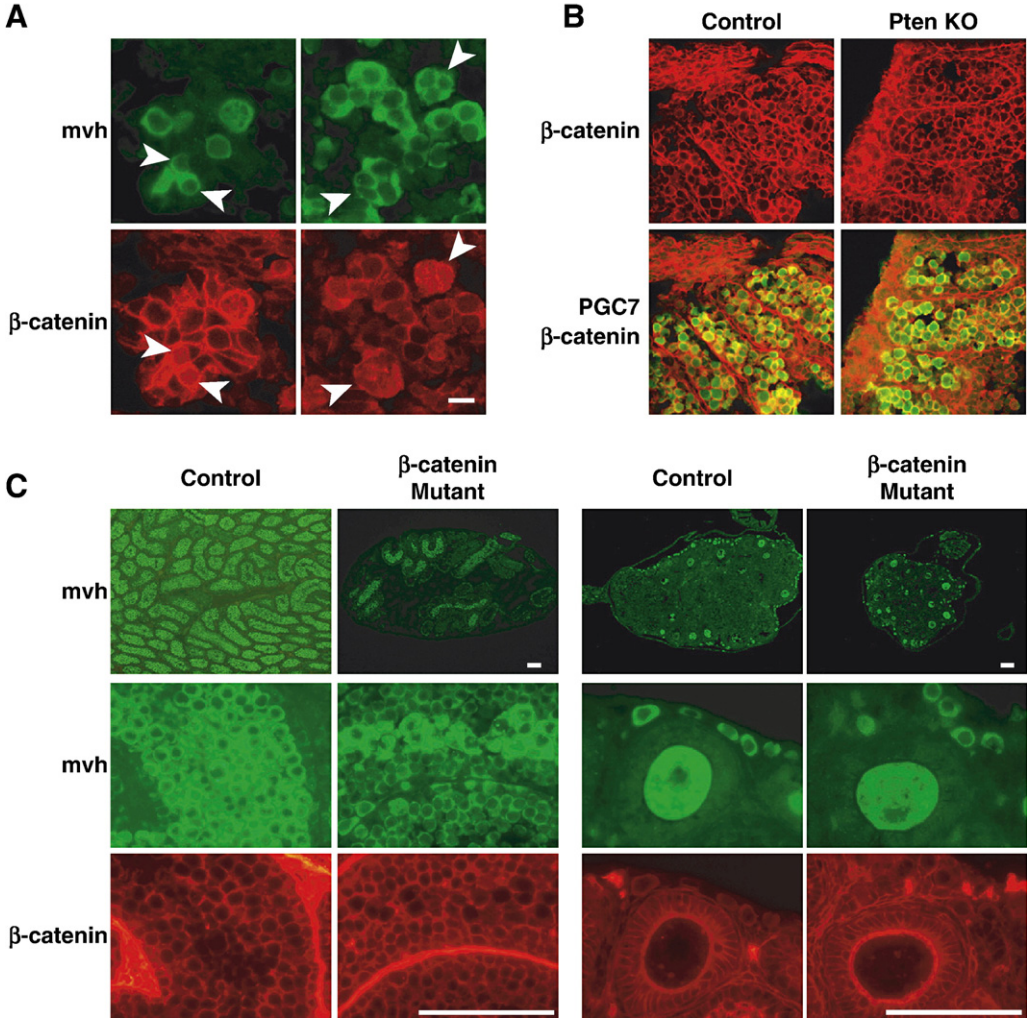

Fig. 3. Germ cell deficiency in the nuclear-localized β-catenin mutant mice. (A) The nuclear accumulation of β-catenin in PGCs of the stabilized β-catenin mutant mice at E13.5. The E13.5 gonads were stained with antibodies against mvh (green) and β-catenin (red) and analyzed under confocal microscopy at 1-μm thickness. The mutant (*β-catenin*$^{floxed\ exon3/+}$; *TNAP-Cre*) mice produce β-catenin protein lacking GSK3β phosphorylation sites from the endogenous *β-catenin*$^{\Delta exon3}$ locus. In the mutant mice, strong nuclear staining was detected in some populations of PGCs (white arrowheads). Scale bar, 10 μm. (B) Subcellular localization of β-catenin in PGCs of the *Pten*-deficient mice at E13.5. The gonads were stained with antibodies against PGC7/Stella (green) and β-catenin (red). β-catenin was not accumulated in the nucleus of PGCs in the *Pten*-deficient mice. (C) Sections of testes (left panels) and ovaries (right panels) at 2 weeks of age were immunostained with antibodies against mvh (green) and β-catenin (red). The sections of central part of the testes and ovaries are shown. The size of gonads was smaller, and the number of germ cells was reduced in the stabilized β-catenin mutant mice. Note that nuclear accumulation of β-catenin was not observed in the germ cells of mutant mice. Scale bar, 100 μm.

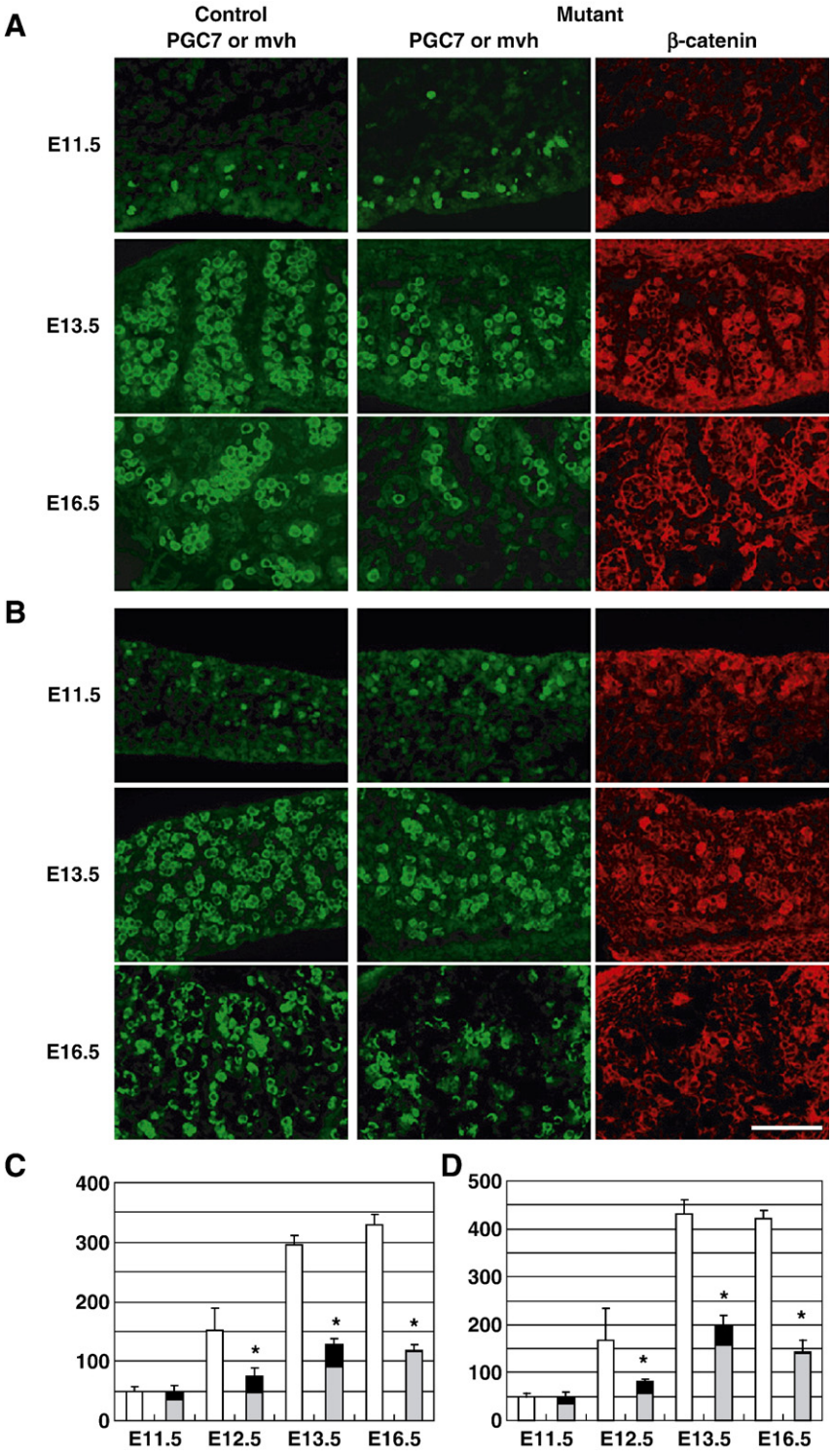

Fig. 4. Development of PGCs in the mutant embryos with stabilized β-catenin. (A, B) Male (A) and female (B) gonads at various developmental stages stained with antibodies against β-catenin (red) and the germ-cell-specific markers (green) PGC7/Stella (E11.5) or mvh (after E12.5). Scale bar, 100 μm. (C, D) The number of germ cells in male (C) and female (D) gonads. Open bars represent the number of PGCs in control mice. Gray and closed bars indicate the number of PGCs in mutant mice that showed β-catenin staining predominantly in the membrane and cytoplasm and in the nucleus, respectively. Values represent the means±SEM and were analyzed using Student's *t* test. At least three mutant mice were analyzed at each developmental stage. After E12.5, the total number of germ cells in the stabilized β-catenin mutant mice was significantly lower than that in control mice (*$p<0.05$ at E12.5 and *$p<0.005$ at E13.5 and E16.5). The percentages of PGCs expressing stabilized β-catenin out of the total PGCs were as follows: 28.4±4.8%, 37.1±8.4%, 29.7±1.0%, and 2.0±0.2% for E11.5, E12.5, E13.5, and E16.5 males, and 27.1±5.7%, 29.1±2.1%, 21.0±0.8%, and 1.4±0.7% for E11.5, E12.5, E13.5, and E16.5 females, respectively (means±SEM).

the resulting *β-catenin*$^{\Delta exon3}$ locus. Significant proportion of the PGCs in the mutant mice exhibited a strong β-catenin signal in the nucleus, as well as in the plasma membrane (Fig. 3A). This indicates that nuclear accumulation of β-catenin is inhibited by GSK3β-mediated degradation in PGCs. We also examined the subcellular localization of β-catenin in *Pten*-

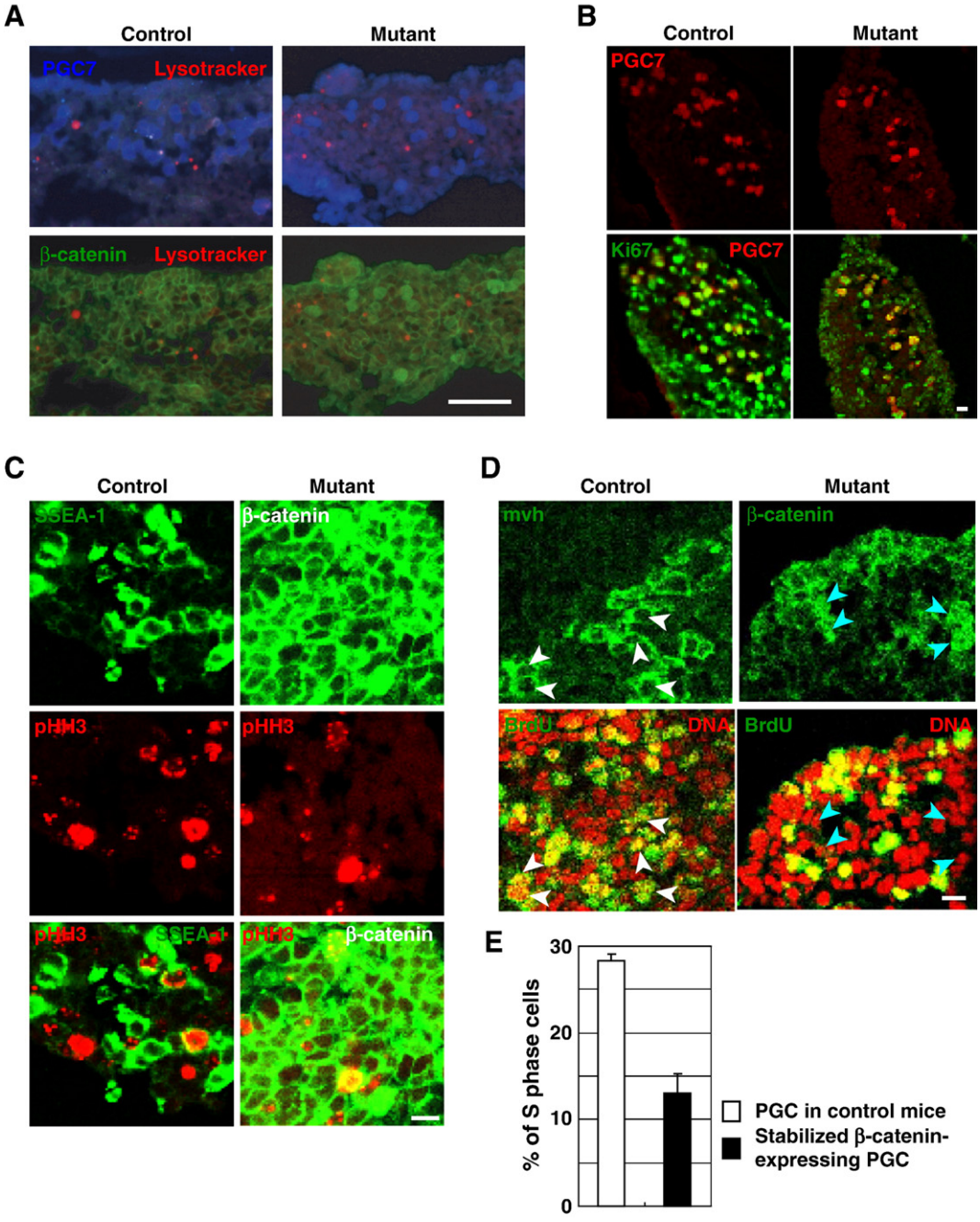

Fig. 5. Apoptosis and cell cycle status of the nuclear-localized β-catenin-expressing PGC. (A) Staining with the apoptosis marker LysoTracker. The E12.0 gonads were incubated with LysoTracker (red) for 30 min. The sections were then stained with anti-PGC7 (blue) and anti-β-catenin (green) antibodies. The upper panels are the merged LysoTracker and PGC7 images, and the bottom panels are the merged LysoTracker and β-catenin images. LysoTracker-positive cells were mainly observed in PGC7-negative somatic cells. Scale bar, 100 μm. (B) Immunostaining with anti-Ki67 antibody. The sections of E12.0 gonads were stained with anti-PGC7 (red) and anti-Ki67 (green) antibodies. The bottom panels are the merged images of PGC7 and Ki67. Almost all PGC7-positive cells were Ki67-positive. Scale bar, 10 μm. (C) Immunostaining with anti-pHH3 antibody. The sections of E12.0 gonads from control mice were stained with anti-pHH3 antibody (red) and SSEA-1 (green). The sections of the mutant mice were stained with anti-pHH3 (red) and anti-β-catenin (green) antibodies. Scale bar, 10 μm. (D) BrdU incorporation assay. Serial sections of E12.0 gonads (4-μm thickness) were stained with anti-BrdU (green; bottom panels). The adjacent sections were stained with anti-mvh (green) or anti-β-catenin (green) antibodies (upper panels). White arrowheads indicate BrdU-positive PGCs in control mice, and blue arrowheads show the nuclear-localized β-catenin-expressing PGCs that were negative for BrdU. Scale bar, 10 μm. (E) The percentage of S phase PGCs in nuclear-localized β-catenin mutant mice. The percentages of BrdU-positive cells per mvh-positive PGC were calculated in control mice. The percentages of BrdU-positive cells per nuclear-localized β-catenin-expressing PGC were calculated in the mutant mice. The percentage of S phase cells was significantly lower in the nuclear-localized β-catenin-expressing PGCs (means ± SEM; $p < 0.05$, Student's t test, $n = 3$).

deficient (*Pten*$^{flox/-}$; *TNAP-Cre*) mice because the nuclear accumulation of β-catenin is linked to the activation of Akt signaling (He et al., 2004; Persad et al., 2001; Tian et al., 2004). However, in the *Pten* mutant mice, β-catenin was localized normally in the plasma membrane and cytoplasm, but not in the nucleus (Fig. 3B).

The number of germ cells decreased in both the testes and ovaries of the 2- to 3-week-old mutant mice (Fig. 3C). At this stage, nuclear localization of β-catenin was not detected in the germ cells. In the β-catenin mutant mice, normal spermatogenesis proceeded in the testes, and primordial and primary follicles containing germ cells were observed in the ovaries. The onset of testicular and ovarian teratomas was not observed in mutant mice of this age. Teratoma formation in adult mice could not be assessed because the mutant mice died by 3 weeks of age, presumably due to Cre-mediated recombination in other tissues, such as the brain and intestine.

Impaired PGC development in the nuclear-localized β-catenin mutant embryos

Next, we analyzed the developmental process of PGCs by co-immunostaining with antibodies against β-catenin and germ cell markers, such as PGC7/Stella, mvh, and SSEA-1. The expression of *TNAP* starts in E7.25 PGCs and continues until around E15.5 (Ginsburg et al., 1990; MacGregor et al., 1995). Analysis of *TNAP-Cre* mice that carried the reporter transgene showed that recombination efficiency was approximately 60% at E13.5 (Lomeli et al., 2000). As similar recombination efficiency was observed in *Pten*-deficient mice (Kimura et al., 2003b), Cre-mediated recombination proceeds gradually and asynchronously in *TNAP-Cre* mice. As shown in Fig. 4, the nuclear accumulation of β-catenin was detected in 15 to 30% of PGCs in both male and female mutant embryos at E11.5, showing asynchronous recombination in TNAP-Cre mice. The total numbers of PGCs were comparable to those of control embryos at this stage. The number of ectopic PGCs did not increase in the mutant embryos, indicating that the decrease in germ cell number was not due to defective migration. In contrast, in the E12.5 to E13.5 embryos, total PGC numbers decreased to about half of the control embryos in which approximately 20–40% of the cells expressed the nuclear-localized β-catenin on average. The total number of germ cells in the newborn mutant mice decreased to about 30% of the control. Therefore, a drastic reduction of the nuclear-localized β-catenin-expressing PGCs occurred at E11.5 to E13.5, which corresponds to the proliferative phase of PGCs.

Aberrant cell cycle progression in the nuclear-localized β-catenin-expressing PGCs

Next, we investigated the mechanism of PGC deficiency. Excess amounts of β-catenin induced cell death in a variety of cell lines and primary cells (Kim et al., 2000; Olmeda et al., 2003). Since the nuclear accumulation of β-catenin was detected in only 20–40% of the PGCs of the mutant mice, we examined the rate of cell death in the nuclear-localized β-catenin-expressing PGCs. When the E12.0 gonads of the mutant mice were stained with the apoptosis marker LysoTracker, few apoptotic cells were detected in the PGCs regardless of the nuclear-localized β-catenin (Fig. 5A). Similar results were obtained when E12.0 gonads were examined using the TUNEL assay (data not shown).

The enhanced expression of β-catenin leads to cell cycle arrest in the G1/G0, G1/S, and G2/M phases (Damalas et al., 2001; Johnston and Edgar, 1998; Mao et al., 2001; Olmeda et al., 2003). To examine whether cell cycle arrest occurred in the mutant mice, antibodies against Ki67 and phospho-histone H3 (pHH3) were used to stain the nuclear-localized β-catenin-expressing PGCs at E12.0. Ki67 is a protein expressed in all phases of the cell cycle, except G0 phase. Almost all the PGCs in the control and stabilized β-catenin mutant mice were positive for the Ki67 antigen (Fig. 5B), indicating that all the PGCs were actively cycling at this stage and that the stabilization of β-catenin did not cause G0 arrest in PGCs. Next, we stained the PGCs with antibody against Ser10 phosphorylated histone H3 (pHH3) which is associated with the condensed chromosomes during G2 and M phases (Fig. 5C). There were no significant differences in the percentages of pHH3-positive cells between the stabilized β-catenin-expressing PGCs in the mutants and the PGCs in control mice (3.0± 1.4% $n=4$ and 3.1±1.8% $n=3$, respectively). Finally, we examined the rate of G1 to S phase transition using the BrdU incorporation assay. The BrdU-positive cells significantly decreased in the nuclear-localized β-catenin-expressing PGCs (Figs. 5D, E). In conclusion, the reduction in PGC number in the mutant mice resulted from the delayed cell cycle progression at the G1/S transition.

Discussion

In this study, we demonstrated that the suppression of Wnt/β-catenin signaling is a prerequisite for normal cell cycle progression in PGCs because the aberrant activation of this signaling resulted in germ cell deficiency by delaying cell cycle progression. Active suppression of Wnt/β-catenin signaling was seen in the analysis of the PGC-specific elimination of exon 3 from the endogenous *β-catenin* locus. In the mutant mice, the mutant β-catenin lacking GSK3β phosphorylation sites was stabilized and accumulated in the nuclei of PGCs. In addition to GSK3β-mediated degradation of β-catenin, the low expression of canonical Wnts in embryonic testes and ovaries supported the suppression of Wnt/β-catenin signaling in PGCs. Consistent with our results, in the transgenic mice carrying a reporter gene under the control of β-catenin/TCF-responsive elements, reporter activity was not detected in gonads (Maretto et al., 2003). In contrast to canonical Wnts, non-canonical Wnts, such as *Wnt-4* and *Wnt-11*, were highly expressed in developing gonads. Wnt-4 is crucial for embryonic oocyte development, as well as for migration of the Leydig cell lineage and the formation of Müllerian ducts (Jeays-Ward et al., 2003; Vainio et al., 1999). Despite the high levels of expression, Wnt-11 and Wnt-5a are not required for germ cell development, but instead regulate the development of the metanephric kidney and female

reproductive tract, respectively (Majumdar et al., 2003; Mericskay et al., 2004).

PGCs proliferate actively from E7.25 to E13.5 and subsequently enter mitotic and meiotic arrest at E13.5 in males and females, respectively (McLaren, 2003; Wylie, 2000). Although the proliferation of PGCs determines the number of founder spermatogonia and oocytes, the mechanisms regulating PGC proliferation in vivo are poorly understood. Of the growth factors that affect the proliferation of PGCs in vitro, SCF is a crucial regulator of PGC proliferation in vivo (Chabot et al., 1988; Geissler et al., 1988). In the SCF and its receptor c-kit of mutant mice, the number of PGCs is severely reduced in the early phase of migratory PGCs. In addition to the SCF/c-kit system, Pin1, a peptidyl-prolyl isomerase, also controls the cell cycle of proliferative PGCs because a lack of Pin1 causes germ cell deficiency as a result of inefficient G1 to S phase transition (Atchison et al., 2003). We demonstrated that inhibition of GSK3β-mediated degradation of β-catenin delayed the G1/S transition in actively proliferating PGCs, consistent with the effects of stabilized β-catenin in other cell types (Damalas et al., 2001; Johnston and Edgar, 1998; Mao et al., 2001; Olmeda et al., 2003). Therefore, suppression of Wnt/β-catenin signaling is required for cell cycle progression of PGCs to ensure an adequate number of germ cells.

The germ cells with nuclear-localized β-catenin gradually disappeared after E13.5. The cell cycle arrest induced by over-expression of β-catenin is frequently followed by apoptosis (Kim et al., 2000; Olmeda et al., 2003). Although increased cell death was not detected during the proliferative phase (Fig. 5A), the nuclear-localized β-catenin-expressing germ cells may have been lost by cell death and have been cleared rapidly by phagocytic activity of gonadal somatic cells. On the other hand, faint band of exon-3-deleted *β-catenin* locus was detectable by genomic RCR of the neonate mutant testis and ovary (data not shown), showing that at least a small number of the germ cells with deletion of exon 3 survived until neonate period. Consistent with this observation, the presence of GSK3β-independent degradation of β-catenin has been reported in oocytes and preimplantation embryos because the oocyte-specific deletion of exon 3 of *β-catenin* did not induce nuclear accumulation of β-catenin in oocytes and preimplantation embryos (Kemler et al., 2004). Similarly, nuclear accumulation of the mutant β-catenin might be prevented in the germ cells after E13.5 independent of GSK3β, perhaps via a mechanism like that involving ubiquitin ligase Siah-1 (Liu et al., 2001; Matsuzawa and Reed, 2001).

Wnt/β-catenin and PI3K signals regulate "stemness" in various stem cell systems (Groszer et al., 2001; Huelsken et al., 2001; Reya et al., 2003; van de Wetering et al., 2002; Zechner et al., 2003). In particular, the pluripotency of ES cells is regulated by both signals (Paling et al., 2004; Sato et al., 2004; Watanabe et al., 2006). In the PGC-specific *Pten*-deficient mice in which PI3K signaling is enhanced, testicular teratomas appeared around E16.5 and EG cell generation increased (Kimura et al., 2003b). It has been reported that activation of PI3K/Akt signaling stabilizes β-catenin (He et al., 2004; Persad et al., 2001; Tian et al., 2004). However, the phenotypes observed in *Pten*-deficient mice did not result from aberrant activation of Wnt/β-catenin signaling because no nuclear accumulation of β-catenin was detected in the *Pten*-deficient mice (Fig. 3B). Consistently, the phenotypes of the stabilized β-catenin-expressing mice differed from those of *Pten*-deficient mice. The early onset of teratomas was not observed in the stabilized β-catenin mice, at least until 3 weeks of age. In conclusion, although Wnt/β-catenin signaling regulates various stem cell systems, the activation of Wnt/β-catenin signaling in PGCs is deleterious for normal PGC development and is not involved in tumorigenesis or the stem cell properties of PGCs.

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References

Atchison, F.W., Capel, B., Means, A.R., 2003. Pin1 regulates the timing of mammalian primordial germ cell proliferation. Development 130, 3579–3586.

Brazil, D.P., Yang, Z.Z., Hemmings, B.A., 2004. Advances in protein kinase B signalling: AKTion on multiple fronts. Trends Biochem. Sci. 29, 233–242.

Chabot, B., Stephenson, D.A., Chapman, V.M., Besmer, P., Bernstein, A., 1988. The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. Nature 335, 88–89.

Chuma, S., Nakatsuji, N., 2001. Autonomous transition into meiosis of mouse fetal germ cells in vitro and its inhibition by gp130-mediated signaling. Dev. Biol. 229, 468–479.

Damalas, A., Kahan, S., Shtutman, M., Ben-Ze'ev, A., Oren, M., 2001. Deregulated beta-catenin induces a p53- and ARF-dependent growth arrest and cooperates with Ras in transformation. EMBO J. 20, 4912–4922.

Geissler, E.N., Ryan, M.A., Housman, D.E., 1988. The dominant-white spotting (W) locus of the mouse encodes the c-kit proto-oncogene. Cell 55, 185–192.

Ginsburg, M., Snow, M.H., McLaren, A., 1990. Primordial germ cells in the mouse embryo during gastrulation. Development 110, 521–528.

Groszer, M., Erickson, R., Scripture-Adams, D.D., Lesche, R., Trumpp, A., Zack, J.A., Kornblum, H.I., Liu, X., Wu, H., 2001. Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo. Science 294, 2186–2189 (electronic publication 2001 Nov. 1).

Harada, N., Tamai, Y., Ishikawa, T., Sauer, B., Takaku, K., Oshima, M., Taketo, M.M., 1999. Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. EMBO J. 18, 5931–5942.

He, X.C., Zhang, J., Tong, W.G., Tawfik, O., Ross, J., Scoville, D.H., Tian, Q., Zeng, X., He, X., Wiedemann, L.M., Mishina, Y., Li, L., 2004. BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. Nat. Genet. 36, 1117–1121 (electronic publication 2004 Sep. 19).

Hendzel, M.J., Wei, Y., Mancini, M.A., Van Hooser, A., Ranalli, T., Brinkley, B.R., Bazett-Jones, D.P., Allis, C.D., 1997. Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma 106, 348–360.

Huelsken, J., Vogel, R., Erdmann, B., Cotsarelis, G., Birchmeier, W., 2001. beta-catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. Cell 105, 533–545.

Jeays-Ward, K., Hoyle, C., Brennan, J., Dandonneau, M., Alldus, G., Capel, B., Swain, A., 2003. Endothelial and steroidogenic cell migration are regulated by WNT4 in the developing mammalian gonad. Development 130, 3663–3670.

Johnston, L.A., Edgar, B.A., 1998. Wingless and Notch regulate cell-cycle arrest in the developing *Drosophila* wing. Nature 394, 82–84.

Kemler, R., Hierholzer, A., Kanzler, B., Kuppig, S., Hansen, K., Taketo, M.M., de Vries, W.N., Knowles, B.B., Solter, D., 2004. Stabilization of beta-catenin in the mouse zygote leads to premature epithelial–mesenchymal transition in the epiblast. Development 131, 5817–5824.

Kim, K., Pang, K.M., Evans, M., Hay, E.D., 2000. Overexpression of beta-catenin induces apoptosis independent of its transactivation function with LEF-1 or the involvement of major G1 cell cycle regulators. Mol. Biol. Cell 11, 3509–3523.

Kimura, T., Ito, C., Watanabe, S., Takahashi, T., Ikawa, M., Yomogida, K., Fujita, Y., Ikeuchi, M., Asada, N., Matsumiya, K., Okuyama, A., Okabe, M., Toshimori, K., Nakano, T., 2003a. Mouse germ cell-less as an essential component for nuclear integrity. Mol. Cell. Biol. 23, 1304–1315.

Kimura, T., Suzuki, A., Fujita, Y., Yomogida, K., Lomeli, H., Asada, N., Ikeuchi, M., Nagy, A., Mak, T.W., Nakano, T., 2003b. Conditional loss of PTEN leads to testicular teratoma and enhances embryonic germ cell production. Development 130, 1691–1700.

Kimura, T., Murayama, K., Nakamura, T., Watanabe, S., Umehara, H., Tomooka, M., Nakano, T., 2005. Testicular teratomas: back to pluripotent stem cells. In: Macieira-Coelho, A. (Ed.), Developmental Biology of Neoplastic Growth. Springer, Heidelberg, pp. 133–150.

Liu, J., Stevens, J., Rote, C.A., Yost, H.J., Hu, Y., Neufeld, K.L., White, R.L., Matsunami, N., 2001. Siah-1 mediates a novel beta-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein. Mol. Cell 7, 361–365.

Logan, C.Y., Nusse, R., 2004. The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. 20, 781–810.

Lomeli, H., Ramos-Mejia, V., Gertsenstein, M., Lobe, C.G., Nagy, A., 2000. Targeted insertion of Cre recombinase into the TNAP gene: excision in primordial germ cells. Genesis 26, 116–117.

MacGregor, G.R., Zambrowicz, B.P., Soriano, P., 1995. Tissue non-specific alkaline phosphatase is expressed in both embryonic and extraembryonic lineages during mouse embryogenesis but is not required for migration of primordial germ cells. Development 121, 1459–1487.

Majumdar, A., Vainio, S., Kispert, A., McMahon, J., McMahon, A.P., 2003. Wnt11 and Ret/Gdnf pathways cooperate in regulating ureteric branching during metanephric kidney development. Development 130, 3175–3185.

Mao, C.D., Hoang, P., DiCorleto, P.E., 2001. Lithium inhibits cell cycle progression and induces stabilization of p53 in bovine aortic endothelial cells. J. Biol. Chem. 276, 26180–26188.

Maretto, S., Cordenonsi, M., Dupont, S., Braghetta, P., Broccoli, V., Hassan, A.B., Volpin, D., Bressan, G.M., Piccolo, S., 2003. Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors. Proc. Natl. Acad. Sci. U. S. A. 100, 3299–3304.

Matsui, Y., Zsebo, K., Hogan, B.L., 1992. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70, 841–847.

Matsuzawa, S.I., Reed, J.C., 2001. Siah-1, SIP, and Ebi collaborate in a novel pathway for beta-catenin degradation linked to p53 responses. Mol. Cell 7, 915–926.

McLaren, A., 2003. Primordial germ cells in the mouse. Dev. Biol. 262, 1–15.

Mericskay, M., Kitajewski, J., Sassoon, D., 2004. Wnt5a is required for proper epithelial–mesenchymal interactions in the uterus. Development 131, 2061–2072.

Moe-Behrens, G.H., Klinger, F.G., Eskild, W., Grotmol, T., Haugen, T.B., De Felici, M., 2003. Akt/PTEN signaling mediates estrogen-dependent proliferation of primordial germ cells in vitro. Mol. Endocrinol. 17, 2630–2638 (electronic publication 2003 Oct. 2).

Moon, R.T., Kohn, A.D., De Ferrari, G.V., Kaykas, A., 2004. WNT and beta-catenin signalling: diseases and therapies. Nat. Rev., Genet. 5, 691–701.

Olmeda, D., Castel, S., Vilaro, S., Cano, A., 2003. Beta-catenin regulation during the cell cycle: implications in G2/M and apoptosis. Mol. Biol. Cell 14, 2844–2860.

Paling, N.R., Wheadon, H., Bone, H.K., Welham, M.J., 2004. Regulation of embryonic stem cell self-renewal by phosphoinositide 3-kinase-dependent signaling. J. Biol. Chem. 279, 48063–48070.

Persad, S., Troussard, A.A., McPhee, T.R., Mulholland, D.J., Dedhar, S., 2001. Tumor suppressor PTEN inhibits nuclear accumulation of beta-catenin and T cell/lymphoid enhancer factor 1-mediated transcriptional activation. J. Cell Biol. 153, 1161–1174.

Resnick, J.L., Bixler, L.S., Cheng, L., Donovan, P.J., 1992. Long-term proliferation of mouse primordial germ cells in culture. Nature 359, 550–551.

Reya, T., Duncan, A.W., Ailles, L., Domen, J., Scherer, D.C., Willert, K., Hintz, L., Nusse, R., Weissman, I.L., 2003. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 423, 409–414.

Sato, M., Kimura, T., Kurokawa, K., Fujita, Y., Abe, K., Masuhara, M., Yasunaga, T., Ryo, A., Yamamoto, M., Nakano, T., 2002. Identification of PGC7, a new gene expressed specifically in preimplantation embryos and germ cells. Mech. Dev. 113, 91–94.

Sato, N., Meijer, L., Skaltsounis, L., Greengard, P., Brivanlou, A.H., 2004. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat. Med. 10, 55–63.

Stevens, L.C., 1967. Origin of testicular teratomas from primordial germ cells in mice. J. Natl. Cancer Inst. 38, 549–552.

Tanaka, H., Pereira, L.A., Nozaki, M., Tsuchida, J., Sawada, K., Mori, H., Nishimune, Y., 1997. A germ cell-specific nuclear antigen recognized by a monoclonal antibody raised against mouse testicular germ cells. Int. J. Androl. 20, 361–366.

Tian, Q., Feetham, M.C., Tao, W.A., He, X.C., Li, L., Aebersold, R., Hood, L., 2004. Proteomic analysis identifies that 14-3-3zeta interacts with beta-catenin and facilitates its activation by Akt. Proc. Natl. Acad. Sci. U. S. A. 101, 15370–15375.

Toyooka, Y., Tsunekawa, N., Takahashi, Y., Matsui, Y., Satoh, M., Noce, T., 2000. Expression and intracellular localization of mouse Vasa-homologue protein during germ cell development. Mech. Dev. 93, 139–149.

Vainio, S., Heikkila, M., Kispert, A., Chin, N., McMahon, A.P., 1999. Female development in mammals is regulated by Wnt-4 signalling. Nature 397, 405–409.

van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A.P., Tjon-Pon-Fong, M., Moerer, P., van den Born, M., Soete, G., Pals, S., Eilers, M., Medema, R., Clevers, H., 2002. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 111, 241–250.

Van Den Berg, D.J., Sharma, A.K., Bruno, E., Hoffman, R., 1998. Role of members of the Wnt gene family in human hematopoiesis. Blood 92, 3189–3202.

Veeman, M.T., Axelrod, J.D., Moon, R.T., 2003. A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. Dev. Cell 5, 367–377.

Watanabe, S., Umehara, H., Murayama, K., Okabe, M., Kimura, T., Nakano, T., 2006. Activation of Akt signaling is sufficient to maintain pluripotency in mouse and primate embryonic stem cells. Oncogene 25, 2697–2707.

Wylie, C., 2000. Germ cells. Curr. Opin. Genet. Dev. 10, 410–413.

Yao, H.H., Whoriskey, W., Capel, B., 2002. Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis. Genes Dev. 16, 1433–1440.

Yoshimizu, T., Sugiyama, N., De Felice, M., Yeom, Y.I., Ohbo, K., Masuko, K., Obinata, M., Abe, K., Scholer, H.R., Matsui, Y., 1999. Germline-specific expression of the Oct-4/green fluorescent protein (GFP) transgene in mice. Dev. Growth Differ. 41, 675–684.

Zechner, D., Fujita, Y., Hulsken, J., Muller, T., Walther, I., Taketo, M.M., Crenshaw III, E.B., Birchmeier, W., Birchmeier, C., 2003. beta-catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system. Dev. Biol. 258, 406–418.